Inhibitors of cell migration

Field of the Invention

5 The present invention concerns peptide compounds, which bind to the α_M integrin I-domain and inhibit its complex formation with proMMP-9, thereby preventing neutrophil migration. The compounds can be used in treatment of inflammatory conditions.

Background of the Invention

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Polymorphonuclear neutrophils (PMNs) constitute the majority of the blood leukocytes and play a pivotal role in acute inflammation by phagocytosing and killing invading microorganisms. The neutrophils contain four granule compartments: azurophilic granules, specific granules, gelatinase granules, and secretory vesicles, defined by their 15 high content of myeloperoxidase (MPO), lactoferrin (LF), gelatinase, and latent alkaline phosphatase, respectively. Proteolytic enzymes, including elastase (1), collagenase (2), and MMP-9 are located in these granules and are important for leukocyte exit from the bone marrow into the circulation and recruitment into the inflammatory sites (3).

20 MMP-9 plays a role in tissue remodeling, tissue repair and wound healing, and is a marker of inflammatory diseases such as rheumatoid arthritis (4) and multiple sclerosis (5). PMNs produce MMP-9 during the late stages of maturation in the bone marrow where it is stored in its latent form (proMMP-9) within the gelatinase granules. Upon cell stimulation, the intracellular granules are rapidly translocated and fused with the plasma 25 membrane. The proMMP-9 zymogen is induced and secreted in response to extracellular stimuli, which initiate specific signalling cascades such as the protein kinase C pathway (6, 7). MMP-9 is also released from human leukocytes after pre-treatment of cells with soluble agonists, such as the complement anaphylatoxin C5a (8) and the tumor necrosis factor-α (TNF-α) (9). Cell adhesion to the extracellular matrix is another known stimulus 30 for secretion of proMMP-9 and other MMPs (10, 11). Selective MMP-9 expression is induced as a result of $\alpha_M\beta_2$ integrin ligation in PMNs (10) and $\alpha_L\beta_2$ integrin ligation in T lymphoma cells (12).

As a result, three different forms of proMMP-9 are released to the extracellular space as detected by zymography: a 92kDa monomer, a 200 kDa homodimer, and a 120 kDa complex of MMP-9 bound to neutrophil gelatinase-associated lipocalin (NGAL), a 25 kDa member of the lipocalin family of transport proteins. Activation of proMMP-9 can

5   be achieved extracellularly by proteinases, or chemically by mercurial compounds or reactive oxygen species (13, 14). Once activated, secreted MMP-9 can be inhibited by the tissue inhibitor of metalloproteinases (TIMP) and α_2-macroglobulin present in the extracellular space. However, TIMP only weakly inhibits the surface MMP-9 of neutrophils (15). Thus, the cell surface localization constitutes yet another level for MMP

10   activity regulation.

Recently, we showed that the proMMP-2 and proMMP-9 gelatinases occur in complex with the $\alpha_L\beta_2$ and $\alpha_M\beta_2$ integrins on the surface of leukemic cells, when the cells are activated by phorbol ester (16, FI 20030923). The β_2 integrins (CD11/CD18) are pivotal

15   for most leukocyte functions (17, 18). Four β_2 integrins have been described: $\alpha_L\beta_2$ which is predominant in leukocytes, $\alpha_M\beta_2$ which is enriched in granulocytes and $\alpha_X\beta_2$ and $\alpha_D\beta_2$ which are predominantly found in monocytes and macrophages. Their cellular ligands are the intracellular adhesion molecules (ICAMs) 1-5, which are members of the immuno-globulin superfamily. The leukocyte integrins need activation to become fully functional

20   (17). T lymphocytes have been most thoroughly studied and activation can occur through the T cell receptor (17, 19) and may involve protein kinase C (20). In granulocytes, $\alpha_M\beta_2$ is known to be located intracellularly in specific granules and upon activation it is translocated to the cell surface (21). Not much is known about the mechanism of translocation and which cellular components are involved.

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We have mapped the major integrin recognition sequence of proMMP-9 to be present in the MMP catalytic domain (16). That sequence was mimicked by phage display peptides discovered by biopanning on the integrin α_M I domain, the most active peptide being ADGACILWMDDGWCGAAG (DDGW). We have studied here the occurrence of the

30   proMMP-9/$\alpha_M\beta_2$ complex in PMNs and its role in PMN migration. We found that the complex between proMMP-9 and $\alpha_M\beta_2$ forms already within the gelatinase granules inside the cell and the complex is translocated to the cell surface upon release of the granules during cell activation. Furthermore, a peptide as small as six amino acids in length derived from the MMP-9 catalytic domain was capable of competing with

proMMP-9 binding to the β_2 integrin. The hexapeptide and DDGW both attenuated PMN migration *in vitro* and *in vivo*, suggesting a role for the MMP-integrin complex in PMN motility.

Summary of the Invention

Experiments with recombinant MMP-9 domains gave further support for our finding that a site interacting with the integrin is present on the MMP-9 catalytic domain and we developed an active I domain binding peptide that was only six residues in length. This peptide, HFDDDE, corresponds to a linear sequence from the MMP-9 catalytic domain and efficiently competed with proMMP-9 binding to $\alpha_M\beta_2$ or its purified I domain. The scrambled peptide had no activity, indicating that the order of the negatively charged amino acids is essential for the activity. Similarly to the phage display-derived DDGW peptide, HFDDDE released cell-bound proMMP-9 and inhibited neutrophil migration *in vitro* and *in vivo*. These results suggest that the proMMP-9/$\alpha_M\beta_2$ complex is important for neutrophil motility but we cannot exclude the possibility that the peptides also affect other β_2 integrin ligands than proMMP-9. However, the fact that DDGW and HFDDDE inhibited the transwell and transendothelial migration of activated neutrophils but not that of resting cells indicates specificity for the action of the peptides. By using CTT, anti-MMP-9 and anti-integrin antibodies, we showed that the peptides inhibited the neutrophil migration that required both proMMP-9 and $\alpha_M\beta_2$. Similarly as with the THP-1 cell line, we thus find that proMMP-9 is a component of the β_2 integrin-directed neutrophil migration at least under these *in vitro* conditions.

Detailed Description of the Invention

We have recently demonstrated that promatrix metalloproteinases, particularly proMMP-9, are potent ligands of the leukocyte β_2 integrins. We studied here the complex formation between proMMP-9 and $\alpha_M\beta_2$, the major MMP and integrin of neutrophils. On resting neutrophils, the proMMP-9/$\alpha_M\beta_2$ complex was primarily detected in intracellular granules, but after cellular activation it became localized to the cell surface as demonstrated by immunoprecipitation and double immunofluorescence. Further indication of the complex formation was that neutrophils and $\alpha_M\beta_2$-transfected L cells, but not the wild-type L cells or leukocyte adhesion deficiency (LAD) cells, bound to

immobilized proMMP-9 or its recombinant catalytic domain in a β_2 integrin-dependent manner. Peptides that bound to the α_M integrin I domain and inhibited its complex formation with proMMP-9 prevented neutrophil migration in a transendothelial assay *in vitro* and in a thioglycolate-elicited peritonitis *in vivo*. These results suggest that the
5   translocating proMMP-9/$\alpha_M\beta_2$ complex may be part of the cell surface machinery guiding neutrophil migration.

In this study, we present evidence that PMNs generate the proMMP-9/$\alpha_M\beta_2$ complex within their intracellular granules and the complex is translocated to the cell surface when
10   the cells are activated with phorbol ester or via inflammatory mediators. Though proMMP-9 is known to localize to the same intracellular granules as the $\alpha_M\beta_2$ integrin, association of proMMP-9 with $\alpha_M\beta_2$ intracellularly has not been shown before. That proMMP-9 is directly able to bind to the α_M integrin I domain suggests that the interaction between endogenous proMMP-9 and $\alpha_M\beta_2$ is direct although we cannot
15   exclude the possibility of accessory molecules. Previously, endogenous neutrophil elastase, proteinase 3, and cathepsin G have all been reported to bind to $\alpha_M\beta_2$. Thus, $\alpha_M\beta_2$ may have a specific carrier function for some proteinases. We found that ICAM-1 or fibrinogen do not compete with proMMP-9 binding and the DDGW peptide inhibitor of the proMMP-9/$\alpha_M\beta_2$ complex is unable to inhibit leukocyte primary adhesion to ICAM-1,
20   fibrinogen or LLG-C4-GST but still inhibits the cell migration. These results suggest that $\alpha_M\beta_2$-bound proMMP-9 is not essential for primary leukocyte adhesion but rather at some other step of cell invasion, perhaps in degradation of the integrin-directed bonds to matrix proteins.

25   By metabolic labelling of THP-1 leukemic cells, we demonstrated that integrin antibodies coprecipitate proMMP-9 within 2h after the [^{35}S]-methionine pulse, at the time when the integrin chains are first clearly visible. These results indicate that the proMMP-9 association is an early event for the integrins and that the immunoprecipitated material does not represent endocytosed or recycling integrins. This is in accordance with the
30   double-immunofluorescence studies showing extensive intracellular colocalization of proMMP-9 and $\alpha_M\beta_2$ in PMNs that have not been subjected to activation. Following PMA-triggered degranulation, we observed dispersion of the staining and the colocalization shifted to the cell surface. Also, the coprecipitation became most intense from the cell surface fraction. These results suggest a rapid translocation of the preformed

proMMP-9/$\alpha_M\beta_2$ complex from the intracellular pool to the cell surface upon activation. This is a more plausible mechanism for the MMP/integrin complex formation than binding of a secreted MMP to the integrin on the cell surface. First of all, the integrin could transport the endogenously-bound proMMP-9 to an appropriate site without competition by extracellular MMP inhibitors and integrin ligands. Secondly, as the I domain of $\alpha_M\beta_2$ does not bind active MMP-9, the integrin could regulate the timing of proMMP-9 activation and release of the active enzyme.

The leukocyte β_2 integrins are involved in leukocyte mobility. Studies with α_M or α_L knockout mice also show the importance of β_2 integrins in mediating leukocyte adhesive, migratory, and phagocytic activities in response to inflammatory stimuli. Leukocytes from patients with the leukocyte adhesion deficiency syndrome type I (LAD-1) have a defective β_2 integrin subunit and cannot migrate properly although they express proMMP-9, indicating that proMMP-9 alone does not confer cell migration ability. We found that LAD-1 cells expressed MMP-9 immunoreactivity at the leading edge, but did not adhere to the immobilized proMMP-9. Very similar results were obtained with wild type L cells which were unable to adhere to the immobilized proMMP-9 but acquired the ability after transfection of $\alpha_M\beta_2$. Experiments with PMNs suggest that proMMP-9 would associate with both the intracellular "inactive" integrin and the extracellular integrin once activated by PMA, C5a or TNFα stimulus. It remains to be determined how (pro)MMP-9 is located at the cell surface in LAD-1 cells in the absence of β_2 integrin. There are a number of other binding proteins reported for MMP-9 in the literature.

The cell migration assays revealed two modes of cell motility: β_2 integrin-dependent that was inhibited by DDGW and other peptides, and β_2 integrin-independent that was not inhibited by the peptides. Thus, it is not surprising that the literature is controversial in terms of the role of proMMP-9 in neutrophil migration. Depending on the experimental models and animal species, some studies have supported protease function in neutrophil migration, whereas others have not. The ability of the cells to show different modes of migration with regard to the stimulus could explain many of the discrepancies. The β_2 integrin- and MMP-independent leukocyte migration may correspond to the observed amoeboid-like movement of leukocytes in 3-dimensional collagen under *in vitro* conditions, which is insensitive to MMP inhibitors.

MMP-9 null mice still show neutrophil migration in thioglycolate-induced peritonitis and *in vitro* transmigration of neutrophils across TNF-α-treated endothelial cells. However, MMPs are known to have overlapping functions and other MMPs could compensate for the loss of MMP-9. We have previously found that proMMP-2 complexes with $\alpha_M\beta_2$ and the studies here show that neutrophil MMP-8 can also bind to purified I domain. The HFDDDE sequence is highly conserved in secreted MMPs and such peptides from many MMPs can bind α_M I domain in a pepspot membrane assay (16, FI 20030923). It remains to be seen which MMP-integrin complexes are functional in the MMP-9 knockout mice. Furthermore, the ability of $\alpha_M\beta_2$ to bind also other proteinases such as elastase and urokinase likely affects neutrophil invasivity.

DDGW and HFDDDE had potent activities *in vivo* in the mouse peritonitis model, but it is unclear to what extent this was due to inhibition of proMMP-9 as both peptides can potentially inhibit other β_2 integrin ligands as well. A subset of β_2 integrin ligands have a DDGW-like sequence and these include, in addition to MMPs, at least complement iC3b and thrombospondin-1. Our results suggest that the proMMP-9/$\alpha_M\beta_2$ complex may be part of the neutrophil's machinery for a specific β_2 integrin-directed movement.

The present invention is thus directed to new peptide compounds, in specific to a peptide compound comprising the hexapeptide motif HFDDDE. Said compounds can be used as pharmaceuticals, which inhibit neutrophil migration. The inhibitory activity was shown both in *in vitro* and *in vivo* experiments. Consequently, the compounds can be used to prevent and treat inflammatory conditions.

The invention thus concerns a compound comprising the hexapeptide motif HFDDDE, and, especially, such a compound for use in inhibiting neutrophil migration, and such a compound for use in prevention and treatment of inflammatory conditions.

The invention is also directed to the compounds of the invention for the manufacture of a pharmaceutical composition for the treatment of conditions dependent on neutrophil migration.

Another embodiment of the invention is a pharmaceutical composition comprising as an active ingredient a compound of the invention, and a pharmaceutically acceptable carrier.

A further embodiment of the invention is a method for therapeutic or prophylactic treatment of conditions dependent on neutrophil migration, comprising administering to a mammal in need of such treatment a neutrophil migration inhibiting compound of the invention in an amount which is effective in inhibiting migration of neutrophils. Specific embodiments of the invention include methods for prophylaxis and treatment of inflammatory conditions.

Abbreviations: HMEC, human microvascular endothelial cell; PMN, polymorpho-nuclear neutrophil; CTT, CTTHWGFTLC peptide; CTT W→A, CTTHAGFTLC peptide; LLG-C4, CPCFLLGCC peptide; DDGW, ADGACILWMDDGWCGAAG peptide; HSA, human serum albumin; KKGW, ADGACILWMKKGWCGAAG peptide; LF, lactoferrin; MPO, myeloperoxidase; NGAL, neutrophil gelatinase-associated lipocalin; GPA, glycol-phorin A, TAT-2: tumor-associated trypsinogen-2.

Description of the Drawings

FIGURE 1A to 1E. Double immunofluorescence staining for $\alpha_M\beta_2$ and proMMP-9 in human neutrophils and LAD-1 cells. Freshly isolated PMNs (**1A, 1B, 1C, and 1D**) from healthy donors and LAD-1 cells (**1E**) were double stained for MMP-9 and $\alpha_M\beta_2$ integrin (see Experimental). Briefly, unstimulated (**1A and 1B**) or PMA-stimulated PMNs (**1C and 1D**) were added to poly-L-lysine-coated coverslips, fixed, and permeabilized (**1A and 1C**) or not (**1B and 1D**). Cells were treated with anti-$\alpha_M\beta_2$ and anti-MMP-9 antibodies followed by staining with FITC-labelled and TRITC-labelled secondary antibodies. (**1E**) Non-permeabilized LAD-1 cells were fixed and stained similarly. Fluorescence was detected by confocal microscopy (**A, B, C, D, and E**; bars: 4.5, 3.4, 7.0, 4.8, and 5.8µm, respectively). The experiments were repeated at least 3 times with similar results.

FIGURE 2A to 2F. Subcellular fractionation of nitrogen-cavitated disrupted neutrophils on a Percoll gradient. Isolated neutrophils were kept on a resting state or stimulated prior to cell lysis. After Percoll gradient centrifugation, fractions were divided into the populations denoted α, $\beta1$, $\beta2$, and γ, respectively. S0, supernatant before or after PMA-stimulation; S1, postnuclear supernatant; S2, cytosolic material. These pooled fractions were assayed for MPO (**2A**), NGAL (**2B**), LF (**2C**), MMP-9 (**2D**), HSA (**2E**), and HLA (**2F**) by ELISA. The experiment was repeated at least 3 times with similar results.

FIGURE 3A to 3D. Subcellular localization of $\alpha_M\beta_2$ and MMP-9 in neutrophils granules.

(3A) Equal amounts of total protein from each granule pool (α, $\beta1$, $\beta2$, and γ-band) were separated by SDS-PAGE and analysed by immunoblotting using polyclonal anti-MMP-9 and the anti-α_M antibody MEM170.

(3B) Gelatinase activity from each pool was detected by gelatin zymography. The positions and molecular masses (kDa) of the bands containing gelatinolytic activity are indicated with arrows.

(3C) Solubilized membrane proteins isolated from each pool were immunoprecipitated with the anti-α_M antibody OKM10 and detected by western blotting using polyclonal anti-MMP-9 or the anti-α_M antibody MEM170.

(3D) THP-1 cells were pulse labeled with [^{35}S]-methionine for 10 min followed by chase for up to 4 h. Cell lysates were incubated with anti-MMP-9, anti-α_M, or control (human IgG) antibodies for 3 h. The immunoprecipitates were visualized by fluorography after 24 h. The positions of proMMP-9 and α_M subunit are marked.

FIGURE 4A to 4D. α_M-I domain binding to recombinant MMP-9 domains.

(4A) Schematic representation of MMP-9 and its recombinant forms produced in *E. coli*.

(4B) ProMMP-9, its recombinant forms or BSA were coated on microtiter wells (80µg/well) and soluble GST-α_M I domain was allowed to bind at the concentrations indicated. The binding was determined by anti-GST monoclonal antibody. The results are means $\pm$ SD from triplicate wells in this and other figures.

(4C) Binding of proMMP-9 to the immobilized GST-α_M I domain was studied in the presence of each peptide at the concentrations indicated. The binding was determined with the anti-MMP-9 antibody GE-213.

(4D) Binding of GST-α_M I domain to the immobilised proMMP-8, proMMP-9, ICAM-1, and fibrinogen was studied with ICAM-1, DDGW or KKGW (50 µM) as competitors. In control wells, GST was added instead of GST-α_M I domain. The experiment was repeated three times with similar results.

FIGURE 5A to 5D. Recognition of recombinant MMP-9 domains by $\alpha_M\beta_2$ integrin-expressing cells. The studied cells were PMNs (5A, 5B, 5C), $\alpha_M\beta_2$ L-cell transfectants (5D), non-transfectants (5D), and LAD-1 cells (5D). PMNs were in resting state or stimulated with PMA (5A, 5C) or C5a or TNFα (5B) before the binding experiment to

proMMP-9 or its domains. Cells were also pretreated with each peptide (50 μM), antibody (20μg/ml) or the α_M I domain as indicated. Unbound cells were removed by washing and the number of adherent cells was quantitated by a phosphatase assay. The experiment was repeated three times with similar results.

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FIGURE 6A to 6D. Blockage of PMN and THP-1 cell migration *in vitro* by gelatinase and β_2 integrin inhibitors. PMNs (1×10^5 in 100 μl) were applied on the LLG-C4-GST or GST coated surface **(6A)** or HMEC monolayer **(6B)** in the absence or presence of peptides (200 μM) or antibodies (20μg/ml) as indicated. PMNs were stimulated with 20

10   nM PMA **(6A)**, HMECs with 50 μM C5a or 10ng/ml TNFα or left untreated **(6B)**. THP-1 cells (5×10^4 in 100μl) were stimulated with 50 nM PMA and applied on the coated surfaces together with each peptide (200 μM) **(6C)**. The cells migrated through transwell filters were stained and counted microscopically. All experiments were reapeated at least twice. **(6D)** Phorbol ester-activated THP-1 cells (5×10^4 in 100μl) were incubated for 16 h

15   at +37°C in the presence or absence of peptides as indicated. The conditioned medium was analyzed by gelatin zymography.

FIGURE 7A to 7D. Inhibition of neutrophil migration to an inflammatory tissue.

(7A) Mice were injected with thioglycolate or PBS intraperitoneally. The peptides were

20   applied intravenuously at the amounts indicated **(A)**. After 3 h, the intraperitoneal leukocytes were harvested and counted. The results show means ± SD of 2 − 4 mice in a group. **(*)** indicates statistical significant difference (p<0.001). The experiment was repeated at least 3 times. The infiltrated neutrophils of mice treated with thioglycolate **(7B)** or PBS **(7C)** were stained with anti-MMP-9 and anti-α_M, as described in the figure 3

25   legend. Fluorescence was studied by confocal microscopy. Bars: 9.1μm and 4.8μm, respectively.

(7D) Gelatinolytic activity of the supernatants from the peritoneal cavities of mice collected as in **(7A)**. Lanes 1-4: samples are from thioglycolate-treated mice; lane 5: a sample from PBS-treated mouse. DDGW, HFDDDE, and DFEDHD were injected

30   intravenously at doses of 0.1, 0.2 and 0.2 mg per mouse. The arrows show proMMP-9 dimer, proMMP-9 and proMMP-2. The experiment was repeated three times with similar results.

The publications and other materials referred to or used herein to illuminate the

35   background of the invention, and in particular, to provide additional details with respect

to its practice, are incorporated herein by reference. The invention will be described in more detail in the following Experimental Section.

Experimental

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Neutrophil preparations and cell lines

PMNs were isolated from peripheral blood anticoagulated in acid-citrate dextrose. Erythrocytes were sedimented by centrifugation on 2% Dextran T-500, and the leukocyte-rich supernatant was pelleted, resuspended in saline and centrifuged on a

10   Lymphoprep (Nyegaard, Oslo, Norway) at 400g for 30 minutes to separate polymorphonuclear cells from platelets and mononuclear cells (22). PMN purity was >95% with typically <2% eosinophils. Cell viability was measured using an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium) bromide assay as instructed by the manufacturer (Roche).

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Human microvascular endothelial cells (HMEC-1) (23), kindly provided by S. Mustjoki (Haartman Institute, University of Helsinki), were grown in RPMI 1640 in the presence of 10% FBS containing 2 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. Human monocytic THP-1 cells were maintained as described (24, 25).

20   Leukocyte adhesion deficiency type-1 (LAD-1) cells, wild type and $\alpha_M\beta_2$-transfected L929 mouse fibroblastic cells were generous gifts from Dr. Jean-Pierre Cartron (INSERM, Paris, France). These cells were maintained as described previously (26) and the $\alpha_M\beta_2$ expression was examined by fluorescence-activated cell sorting (FACS, Becton Dickinson, San Jose, CA).

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Antibodies and other reagents

The monoclonal antibodies MEM170 and OKM10 are against the integrin α_M subunit (25). The monoclonal anti-MMP-9 antibody (GE-213) was obtained from LabVision (Fremont, CA) and polyclonal MMP-9 from Santa Cruz Biotechnology (Santa Cruz, CA).

30   We also used the previously reported affinity purified antibodies against MMP-9 (3). As monoclonal antibody controls, we used a mouse IgG (Silenius, Hawthorn, Australia) and anti-glycophorin A (GPA) (ATCC). Anti-trypsinogen-2 (TAT-2) antibody was a rabbit polyclonal antibody control (27). The peroxidase-conjugated anti-GST mAb was from Santa Cruz Biotechnology. A rat antibody against the mouse α_M integrin (MCA74) and a

35   FITC-conjugated anti-rat (Fab')₂ were purchased from Serotec (Oxford, UK). The

peptides CTT, W→A CTT, LLG-C4, DDGW, and KKGW have been described earlier (16, 28). The HFDDDE and DFEDHD peptides were custom-made by Neosystem (Strasbourg, France). ProMMP-8 and proMMP-9 were obtained from Calbiochem and Roche, respectively. Diisopropyl fluorophosphate was from Aldrich Chemical Company Inc. (Steinheim, Germany). Human C5a and recombinant TNF-α were purchased from Calbiochem (Biosciences, Inc. La Jolla, CA) and Sigma-Aldrich (St. Louis, MO), respectively.

Subcellular Fractionation

PMNs were suspended in Krebs-Ringer phosphate (130 mM NaCl, 5 mM KCl, 1.27 mM MgSO$_4$, 0.95 mM CaCl$_2$, 5 mM glucose, 10 mM NaH$_2$PO$_4$/Na$_2$HPO$_4$, pH 7.4) at 3 x 10^7 cells/ml. PMNs were incubated with or without phorbol myristate acetate (PMA; 2 μg/ml) at +37°C for 15 minutes, then with 25 mmol/L diisopropyl fluorophosphate for 5 min on ice and the supernatant (S0) was collected. Granule fractions were purified as previously described (29). Briefly, PMNs were disrupted by nitrogen cavitation and cellular debris were removed by centrifugation. The resulting postnuclear supernatant (S1) was applied on a 3-layer Percoll gradient (1.050/1.090/1.120 g/ml) and centrifuged at +4°C for 30 minutes. Fractions 1 to 6, 7 to 12, 13 to 18, and 19 to 24 (1 ml each) were collected and pooled in 4 distinct groups. The clear cytosol (S2) was present in the last fractions (25 to 30). Aliquots were tested for the presence of marker proteins corresponding to individual compartments (indicated in parenthesis): MPO (α band/azurophil), LF (β1 band/specific), gelatinase (β2 band/gelatinase), albumin (γ band/secretory vesicles and plasma membranes) (21). Protein levels were determined using sandwich ELISA assays.

Gelatin zymography, immunoprecipitation, and immunoblotting

Granules fractions were lysed on ice for 15 min with 1% (v/v) Triton-X-100 in phosphate buffered saline (PBS), and the lysate was clarified by centrifugation for 10 min at +4°C. The lysates were analyzed by gelatin zymography on 8% SDS-polyacrylamide gels containing 0.2 % gelatin (27). Before immunoprecipitation, the lysate was precleared by incubating for 30 min at +4°C with protein G-Sepharose. After centrifugation, the supernatant was subjected to immunoprecipitation with polyclonal anti-MMP-9, or monoclonal anti-α$_M$ (OKM-10) antibodies. After incubation at +4°C for 1 h together with protein G-Sepharose, immunocomplexes were pelleted and washed three times with

Triton X-100 lysis buffer and once with PBS. Following solubilization in Laemmli sample buffer with 2-mercaptoethanol, the samples were electrophoresed on 4-15% gradient SDS-PAGE gels (Bio-Rad laboratories, Hercules CA) and transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) by semidry electrophoresis at 15 V for 30 min. Non-specific binding was blocked by soaking the membrane in 5 % milk powder in PBS containing 0.05 % Tween20 at +4°C overnight. The membrane was incubated with a monoclonal α_M (MEM170) antibody (10 µg/ml) for 2 h at room temperature followed by horseradish peroxidase-conjugated rabbit anti-mouse IgG (1:1000-dilution; DAKO A/S, Copenhagen, Denmark) at 25°C for 30 min. After several washes, the blot was developed with the Enhanced ChemiLuminescence system (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The membranes were stripped of bound antibodies and reprobed with a polyclonal anti-MMP-9 antibody. An appropriate secondary antibody was used. The membranes were stored in TBS at +4°C after each immunodetection.

Expression and purification of integrin I domains and MMP-9 recombinant proteins

GST-α_M and GST-α_L I domain fusion proteins were expressed and purified as described previously (30). GST was cleaved from the α_M I domain with thrombin (Sigma) and the I domain was purified by ion exchange chromatography on a Mono S HR5/5 column using the FPLC system (Pharmacia). The purification of ΔMMP-9 and FnII domains will be described elsewhere (33). The purity of recombinant proteins was checked by SDS-PAGE.

Interactions between MMP-9 domains and α_M I domain

ICAM-1, fibrinogen, proMMP-8, proMMP-9 or the recombinant domains (0.5 µg/well in PBS) were coated on plastic 96-well plates at +4°C for 16 h and the wells were blocked with 3 % bovine serum albumin (BSA) in PBS for 2 h at room temperature. Binding of the GST-α_M I domain was determined essentially as described in the first priority application. In the reverse assay, GST-α_M I domain was coated and binding of proMMP-9 was determined using the GE-213 antibody. Competitor peptides were preincubated with the α_M I-domain for 20 minutes before the experiment.

Metabolic radiolabeling and immunoprecipitation

Non-activated or PMA-activated (50 nM) THP-1 cells (1 x 10^7) were subjected to biosynthetic labeling using [^{35}S]-methionine (31). Cells were suspended in methionine-free medium containing 10% dialyzed, heat-inactivated fetal calf serum and were pulsed-

5   labeled with 50 µCi/ml of [^{35}S]-methionine at +37°C for 10 min. The cells were rapidly washed and further incubated in a complete medium containing 10 % FCS at +37°C for indicated time points. The labeling was stopped by pelleting the cells and adding 2 ml of cold PBS at 3 different time points (30 min, 2 h, and 4 h, respectively). After washings, the cells were lysed with a buffer containing 1% Triton X-100, 10 µg/ml of aprotinin, 10

10   µg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride in PBS, clarified by ultracentrifugation and precleared with protein G-Sepharose. The lysate was immuno-precipitated with affinity purified rabbit anti-MMP-9 and monoclonal α_M (OKM-10) (3 µg/ml). A human IgG$_1$ was a control antibody. After one-hour incubation at +4°C together with protein G-Sepharose, immunocomplexes were pelleted, washed and run on

15   7.5 % SDS-PAGE gels. The gels were treated with an enhancer (Amplify, Amersham Biosciences), dried on a filter paper and exposed to Kodak X-Omat AR film at –70°C for a week.

Immunofluorescence staining

20   PMNs and LAD-1 cells were treated with 20 nM PMA at +37°C for 15 min or left untreated, and then allowed to attach to poly-L-lysine coated cover slips, fixed in 2.5% paraformaldehyde in the presence or absence of 0.1% Triton X-100 at +25°C for 10 min followed by several washings. The cells were blocked with 20 % (v/v) rabbit serum and 3% BSA in PBS at room temperature for 30 min. The cells were incubated with rabbit

25   anti-MMP-9 polyclonal and mouse anti-α_M (MEM170) antibodies (1:250 dilution). After washing with PBS, the secondary antibodies, rhodamine (TRITC)-conjugated anti-rabbit or FITC-conjugated anti-mouse (Fab')$_2$ (DAKO) were incubated at a 1:500 dilution for 30 min. The samples were mounted and slides were kept in the dark at +4°C. Cellular distribution of $\alpha_M\beta_2$ and MMP-9 was examined by fluorescence microscopy and confocal

30   microscopy (Leica multi band confocal image spectrophotometer), equipped with 63 x magnification oil-immersion objective and a Leica TCS SP2 scan unit.

Cell adhesion

MMP-9 proteins (200 nM in PBS) were coated at +4°C for 16 h and the microtiter wells were blocked with 3 % BSA in PBS for 1 h at room temperature. The $\alpha_M\beta_2$-integrin L-cell transfectants and PMNs ($1x10^5$ cells/well) were suspended in RPMI medium supplemented with 2mM $MgCl_2$ and 0.1% BSA and activated with PMA (20nM) for 20 min, or with C5a (50nM) or TNF-α (10nM) for 4h at +37°C. The L926 wild type and LAD-1 cells were used as controls. The cells were treated with the indicated antibody (20 μg/ml) or peptide (50 μM) at +37°C for 30 min, washed twice with serum-free medium and incubated in the microtiter wells at +37°C for 30 min. The wells were washed with PBS, and the number of adherent cells was quantitated by a phosphatase assay (25).

Cell migration

Cell migration was conducted using Costar 24-transwell migration chambers with a 3 μm pore size for PMNs and 8 μm for THP-1 cells. To study β_2 integrin-directed migration, the chamber membrane was coated on both sides with LLG-C4-GST integrin ligand (40μg/ml) or GST as a control and blocked with 10% serum-containing medium (16). To study transendothelial migration, confluent HMECs ($4x10^5$ cells/well) were grown on the upper side of the gelatin-coated membrane for 5 days. Culture medium was changed after 3 days. After washing the HMEC layers twice with PBS, chemotactic activation was carried out by adding C5a (50nM), TNF-α (10ng/ml), or medium alone to the lower compartment at +37°C for 4 h. Cultures were then washed again twice to remove all agents. PMNs or THP-1 cells were preincubated with the peptide inhibitor or antibody studied for 1 h before transfer to the upper compartment ($1x10^5$ cells in 100μl RPMI/0.1 % BSA or the complete 10 % FCS-containing medium). PMNs were allowed to migrate for 2 h through the LLG-C4-GST coated membrane and for 30 min through the HMEC monolayer. THP-1 cells were allowed to migrate for 16 h. The non-migrated cells were removed from the upper surface by a cotton swab and the cells that had traversed the filters were stained with crystal violet and counted.

Mouse inflammation model

Balb/c mice at the age of 31-32 weeks were injected intraperitoneally with 3% (w/v) thioglycolate in sterile saline (32). Peptides (5-500μg in 100μl) were introduced intravenously through the tail vein. Animals were euthanized after 3 h and the peritoneal cells were harvested by injecting 10 ml of sterile PBS through the peritoneal wall. Red

blood cells present in the lavage fluid were removed by hypotonic lysis. Cells were centrifuged and resuspended in 1 ml of sterile 0.25% BSA/Krebs-Ringer. The supernatants were also collected and analysed by gelatin zymography. The number of neutrophils was determined following staining with 0.1% crystal violet and using a light

5   microscope equipped with a x 100 objective. For immunofluorescence staining, cells were allowed to bind to poly-L-lysine coated cover slips, fixed with 2.5% paraformaldehyde in PBS at +4°C for 30 min followed by several washings. The Fc receptors were blocked in the presence of 20% of rabbit serum and 3% BSA in PBS. The cells were then incubated with anti-MMP-9 polyclonal and α_M monoclonal (MCA74)

10 antibodies for 30 min. After washing with PBS, the secondary antibodies, rhodamine (TRITC)-conjugated anti-rabbit or FITC-conjugated anti-rat (Fab')$_2$ were incubated for another 30 min. The samples were examined with a confocal microscope. The animal studies were approved by an ethical committee of Helsinki University.

15 **Statistical analysis**

Results were analysed using the F-test (ANOVA) and subsequently, if significant differences between groups occurred, they were subjected to Duncan's Multiple Range test. The program used was SPSS for Windows release 8.0.

20 **Effect of peptides on gelatinase release from cells**

THP-1 cells (50 000/100µl) were incubated in serum-free RPMI for 16 h in the presence or absence of peptides (200 µM) as described in the text. The supernatants from THP-1 cells and mouse intraperitoneal fluid were analysed by gelatin zymography. Gelatinolytic activity was quantified by densitometric scanning.

25

Results

Intracellular formation of the proMMP-9/$\alpha_M\beta_2$ complex

Staining of resting PMNs with α_M and MMP-9 antibodies showed an intense intracellular colocalization after permeabilization with Triton X-100 (Fig. 1A). In non-permeabilized

30 cells, no such a colocalization was observed and little if any MMP-9 was present on the cell surface (Fig. 1B). After PMA-treatment to cause exocytosis of intracellular granules, the intracellular staining decreased and $\alpha_M\beta_2$ integrin and MMP-9 colocalized to the cell surface (Fig. 1C-D). Similar results were obtained when exocytosis was triggered with C5a (data not shown). As a control for double immunofluorescence staining, we used a B

cell-derived LAD-1 cell line lacking $\alpha_M\beta_2$ (Fig. 1E). After PMA stimulation, the LAD-1 cells secreted proMMP-9 as detected by zymography analysis and it was expressed on the cell surface but the distribution differed from those of neutrophils, MMP-9 localizing to the leading edge.

The intracellular colocalization of proMMP-9 and $\alpha_M\beta_2$ suggested the formation of the proMMP-9/$\alpha_M\beta_2$ integrin complex in the PMN granules before translocation to the cell surface. We purified the azurophilic (α-band), specific (β1-band), gelatinase (β2-band), and secretory vesicles, including the plasma membranes (γ-band) on a three-step discontinuous Percoll gradient from PMA-treated and non-treated cells. The purity of each fraction was assayed by granule-specific markers. MPO was used as a marker for azurophil granules; LF and NGAL for specific granules; MMP-9 for gelatinase granules; human serum albumin (HSA) for secretory vesicles; and human leukocyte antigen (HLA) for plasma membranes (Fig. 2). PMA induced the release of the majority of the granule markers to the extracellular milieu, whereas MPO was only partially released from azurophil granules. Both NGAL and LF were discharged from the specific granules into the supernatant (S0) by 75% and 90%, respectively. Similarly, the MMP-9 content decreased by 90% in the gelatinase granules and increased in the S0 supernatant. HSA from the secretory vesicles was discharged by 85% and detected in large amounts in S0 supernatant. HLA, a marker of the plasma membrane, remained relatively constant. The levels of HSA, NGAL, LF, and MMP-9 were substantially decreased in the postnuclear supernatants (S1) after cell activation. The cytosolic fraction (S2) was devoid of these markers, indicating that the subcellular fractionation led to the isolation of intact granules.

Immunoblot analysis of the granule fractions showed that proMMP-9 was distributed in the subcellular fractions in a similar way as the $\alpha_M\beta_2$ integrin (Fig. 3A). In resting PMNs, the major proportion of proMMP-9 and $\alpha_M\beta_2$ was found in the β2-band, lesser amounts being present in the β1- and γ-bands. In accordance with the immunofluorescence studies, PMA caused a depletion of the β2-band contents. Analysis of the fractions by gelatin zymography similarly showed that PMA decreased the amount of the intracellular proMMP-9 monomer and dimer and its NGAL complex (Fig. 3B). The proMMP-9 zymogen was found in the γ band, representing secretory vesicles and plasma membranes, and in the extracellular milieu (S0).

In the non-activated PMNs, the α_M integrin antibody OKM-10 immunoprecipitated the 165kDa α_M-chain from the β1-, β2-, and γ-bands. The 92 kDa proMMP-9 co-precipitated from the β2-band (Fig. 3C). After PMA-stimulation of cells, the α_M chain was

5 immunoprecipitated from the β1- and γ-bands but not anymore from the β2-band. The integrin antibody co-precipitated proMMP-9 only from the γ-band. Addition of soluble α_M I-domain prevented the co-precipitation.

The biosynthesis of an endogenous complex between proMMP-9 and $\alpha_M\beta_2$ integrin was

10 investigated in the THP-1 leukemic cell line, which is amenable for such studies. The complex was detected at 2 h and 4 h time points by immunoprecipitation from [^{35}S]-methionine pulsed cells (Fig. 3D, lanes 5 and 8). The OKM10 antibody coprecipitated the α_M chain and proMMP-9. The α_M chain was only weakly seen in the immunoprecipitates with anti-MMP-9 antibodies (lanes 4 and 7), possibly because of a large excess of

15 unliganded proMMP-9. A control antibody did not coprecipitate α_M and proMMP-9 (lanes 3, 6, and 9).

Peptide inhibitors of the proMMP-9/$\alpha_M\beta_2$ complex prevent neutrophil migration

In our previous study, pepspot analysis located the integrin interactive site of proMMP-9

20 to a 20-amino acid long sequence present in the catalytic domain, QGDAHFDDDELWSLGKGVVV (see the first priority document). Further screening by the pepspot system has indicated that sufficient integrin binding activity is achieved by truncating this sequence to a hexapeptide, HFDDDE (data not shown). To confirm that such a short sequence is the bioactive site of proMMP-9, we first prepared bacterially

25 expressed recombinant domains of MMP-9 (Fig. 4A). ΔMMP-9 is composed of the prodomain (Pro) and the catalytic domain but lacks the hemopexin domain. The fibronectin type II repeats (FnII) were also produced as a separate recombinant protein as this is an important substrate-binding region. The procatalytic domain construct ΔMMP-9 bound the α_M I domain nearly as efficiently as the wild type proMMP-9 (Fig. 4B). FnII

30 protein almost lacked activity. The HFDDDE peptide identified by the solid-phase pepspot analysis was highly active when made by peptide synthesis and inhibited proMMP-9 binding to the α_M I domain with an IC$_{50}$ of 20 μM (Fig. 4C). The bound proMMP-9 was determined with the GE-213 antibody, which recognizes an epitope of the FnII domain (data not shown). A scrambled peptide DFEDHD with the same set of

negatively charged amino acids was inactive. HFDDDE was equally potent as DDGW, the α_M I domain-binding peptide discovered by phage display. KKGW, the control peptide for DDGW, was without effect. As the HFDDDE sequence is highly conserved in the members of the MMP family, we also examined the α_M I domain binding to human

5   neutrophil collagenase, MMP-8. I domain showed a similar DDGW-inhibitable binding to proMMP-8 as to proMMP-9 (Fig. 4D). ICAM-1 and fibrinogen did not compete with either proMMP, implying different binding sites for the matrix proteins and proMMPs in the I domain.

10   After integrin activation, PMNs exhibited an ability to adhere on proMMP-9. PMA-stimulated PMNs bound to microtiter well-coated ΔMMP-9 nearly as strongly as to proMMP-9 (Fig. 5A). Stimulation of PMNs with C5a or TNF-α gave similar results PMN adherence increasing by 3-fold (Fig. 5B). The FnII domain did not support PMN adhesion. PMN adherence was inhibited by HFDDDE (50μM), DDGW (50μM), the

15   soluble α_M I domain and the MEM170 antibody (Fig. 5C), indicating β_2 integrin-directed binding. The control peptides (DFEDHD, KKGW) and an irrelevant monoclonal antibody (anti-GPA) had no effect. The CTT peptide, but not the W→A CTT control peptide lacking MMP inhibitory activity, binds to the MMP-9 catalytic domain (unpublished results) and also inhibited the PMN adherence. MMP-9 antibodies inhibited

20   partially.

We also examined $\alpha_M\beta_2$–transfected L cells. The $\alpha_M\beta_2$ L-cell transfectants bound to proMMP-9 and ΔMMP-9 similarly as PMNs did and the I domain ligands and MMP-9 inhibitors attenuated the binding (Fig. 5D). The transfected cells also showed a weak

25   adherence to FnII domain, but the studied peptides and antibodies did not inhibit this binding. Wild type L cells or LAD-1 cells showed no binding to proMMP-9 or its domains.

The *in vitro* migration of PMNs was studied on transwell filter assays. Coating with the

30   artificial β_2 integrin ligand LLG-C4-GST renders cell migration dependent on the β_2 integrins (16, 25). The migration of PMA-activated PMNs was 5-fold in the LLG-C4-GST substratum in comparison to GST substratum (Fig. 6A). HFDDDE (200μM) inhibited the migration of PMA-stimulated cells but not the basal migration of non-activated cells. DDGW, CTT, MEM170 (20μg/ml) and polyclonal anti-MMP-9

(20µg/ml) worked similarly, affecting the migration of the PMA-activated cells only. Control peptides and an antibody control (anti-TAT-2) had no effect. Similar results were obtained in a transendothelial migration assay (Fig. 6B). Chemotaxis with C5a or TNF-α increased PMN transmigration by 5-10 fold and inhibition was obtained by DDGW,

5   HFDDDE, and CTT but not with the control peptides. Similarly, α_M and MMP-9 antibodies inhibited but an antibody control (anti-GPA) did not. We also examined the effects of peptides on THP-1 leukemia cell migration through the LLG-C4-GST coated transwell filters. The results were the same as for PMNs. HFDDDE, DDGW, and CTT inhibited THP-1 migration and the control peptides did not (Fig. 6C).

10

Previous studies with the DDGW peptide showed that it can release proMMP-9 from THP-1 cells (FI 2003 0923). We found that the HFDDDE peptide also released proMMP-9 but was less effective than DDGW (Fig. 6D). The scrambled peptide did not induce the release of proMMP-9. Under the 16 h incubation time, the peptides had no effect on the

15   secretion of proMMP-2.

To study neutrophil migration *in vivo*, we used a mouse model of thioglycolate-induced peritonitis. The cells that infiltrated into the peritoneal cavity within 3 h after thioglycolate irritant were judged to be predominantly PMNs by crystal violet staining.

20   The DDGW and HFDDDE peptides had potent *in vivo* activities in this inflammation model (Fig. 7A). An intravenous tail injection of DDGW or HFDDDE inhibited the intraperitoneal accumulation of PMNs. The KKGW and DFEDHD peptides used as controls had no effect. The effects of DDGW and HFDDDE were concentration-dependent and up to 90 % inhibition was obtained by doses of 50µg and 500µg per

25   mouse, respectively. DDGW was active even at 5µg given per mouse corresponding to an effective dose of 0.1mg/kg mouse tissue. Approximately 20-fold more PMNs were present intraperitoneally after thioglycolate-stimulus in comparison to the PBS control. The collected inflammatory PMNs stained positively for the proMMP-9/$\alpha_M\beta_2$ complex by double immunofluorescence (Fig. 7B). The cells collected after PBS injection lacked the

30   complex; they expressed the integrin but had no cell-surface MMP-9 (Fig. 7C). Zymography analysis of the supernatants from the collected intraperitoneal fluid showed that thioglycolate induced elevated levels of gelatinases in comparison to PBS (Fig. 7D). DDGW and HFDDDE, but not the scrambled peptide, prevented the increase in gelatinase levels in accordance with the inhibition of cell migration.

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